The Veliger 49(2):101–104 (July 2, 2007)

THE VELIGER

Effects of Increased Salinity on Survival and Lipid Composition of *Helisoma trivolvis* (Colorado Strain) and *Biomphalaria glabrata* in Laboratory Cultures

DANIELLE L. MARTIN,[1] BERNARD FRIED[2,*] AND JOSEPH SHERMA[1]

[1] Department of Chemistry and [2]Department of Biology, Lafayette College, Easton, Pennsylvania 18042, USA

Abstract. High performance thin layer chromatography (HPTLC) was used to study the effects of increased salinity on the lipid composition of the digestive gland-gonad complex (DGG) of *Biomphalaria glabrata* (NMRI strain) and *Helisoma trivolvis* (Colorado strain) snails. Both species of snails were maintained in laboratory cultures containing various dilutions of artificial ocean water (AOW) for up to 2 weeks. Controls consisted of snails maintained identically in deionized water (DI). Both species of snails maintained in AOW at a salinity of 20% (196 mOsmol/kg) showed no significant changes in the concentrations of the major neutral lipid and phospholipid classes compared to snails maintained in DI. Apparently, the ability to survive for 2 weeks at 20% salinity had no noticeable effect on the concentrations of the depot or structural lipids associated with the DGG of these snails. Snails of both species were dead at 100, 50 and 25% salinities within 90 min. At a salinity of 20%, 3 of 8 *B. glabrata* and 4 of 8 *H. trivolvis* survived for 2 weeks; at 10% salinity, 6 of 8 *B. glabrata* and 7of 8 *H. trivolvis* survived for 2 weeks. Most snails of both species survived for 2 weeks in DI. The ability of planorbid snails to survive relatively high salinities has implications for the spread of larval trematodiases to endemic areas where salinities may be elevated.

INTRODUCTION

Increased salinity in fresh water lakes and ponds is recognized as a serious environmental problem worldwide (Williams, 1987). The question of how large an increase in salinity can be tolerated by most fresh water snails has not been well explored. In our laboratory, we use two species of fresh water snails for various biological and chemical studies. These snails are an NMRI strain of the medically important planorbid *Biomphalaria glabrata* (Say, 1816) and a Colorado strain of the economically important planorbid *Helisoma trivolvis* (Say, 1816). Schneck & Fried (2005) recently described the growth of both of these snail species under the same laboratory conditions.

Our laboratory has examined the effects of various parameters on the lipid content of *B. glabrata* and *H. trivolvis*, i.e., larval trematode parasitism, different diets, snail age, starvation, and estivation. As reviewed in Bandstra et al. (2006a), alterations in lipids, i.e., increase or decrease in the concentrations of particular lipid classes, occur as a function of some of the aforementioned parameters. With this in mind, one purpose of our study was to determine the effects of increased salinity on the lipid content of *B. glabrata* and *H. trivolvis*. Because information on the tolerance of planorbid snails to increased salinities is relatively sparse, another purpose of this study was to examine the effects of increased salinity on the survival of *B. glabrata* and *H. trivolvis* in the laboratory.

* Corresponding author, e-mail: friedb@lafayette.edu

MATERIALS AND METHODS

Stock cultures of *B. glabrata* (NMRI strain) and *H. trivolvis* (Colorado strain) were maintained in the laboratory as described in Schneck & Fried (2005). Adult snails (11 ± 1 mm in shell diameter) of both species were maintained, 4 or 5 snails per culture, in 8.5 cm diameter finger bowls each containing 100 ml of different concentrations of artificial ocean water (AOW) at 22 ± 1°C. The AOW was purchased as a prepared concentrate of artificial salts (Instant Ocean) from Aquarium Systems (Mentor, OH). A stock solution of AOW was made using 43.13 g of Instant Ocean in 1 L of deionized (DI) water to represent full strength sea water with an osmolality of 960 mOsmol/kg. The full strength sea water was diluted using DI water to obtain dilutions of 50, 25, 20, 15, 10, and 5% sea water with osmolalities of 499, 251, 196, 151, 100, and 40 mOsmol/kg, respectively. The osmolalities of the AOW solutions were measured using a Wescor Inc. (Logan, UT) vapor pressure osmometer. For both the survival and lipid–high performance thin layer chromatography (HPTLC) studies, a group of 4 snails of each species was maintained in separate cultures at most salinities for up to 2 weeks. Survival

data determined the salinity that was used in the HPTLC-lipid studies. Cultures were fed romaine lettuce leaves ad libitum and the water was changed every two days. Snails were examined daily for 1 to 14 days after the cultures were set up. Dead snails were removed from the culture on the same day they died. Snails that did not react to mechanical probing were considered dead. Controls for the salinity studies consisted of snails maintained identically but in DI.

For HPTLC analysis, snail digestive gland-gonad complexes (DGGs) were obtained and used as described in Ong et al. (2006). Three DGGs of each snail species (n = 3) maintained in 20% AOW and DI water were prepared for the neutral lipid analysis, and three snail DGGs of each species (n = 3) for analysis of the phospholipids. The use of the 20% AOW was based on the relatively good survival of both snail species for 2 weeks at this salinity (see Results for further information). The blotted wet weight of the *H. trivolvis* snails ranged from 9.8–19.7 mg and the *B. glabrata* ranged from 8.0–23.9 mg. Each sample consisted of a single DGG and was prepared in a tissue grinder (15 mL, KT885300-0015, VWR International, Inc., West Chester, PA, USA) using chloroform-methanol (2:1) with a volume that was twenty times the weight of the sample. After homogenization, the supernatant was filtered through cotton, and a volume of Folch wash (0.88% KCl) was added that was ¼ that of chloroform-methanol (2:1) used for lipid extraction. The upper hydrophilic layer was removed using a Pasteur pipet and discarded; the lower lipophilic layer was dried under nitrogen gas in a water bath (40–60°C). Residues were reconstituted with 80–200 µL of chloroform-methanol (2:1), the exact volume chosen based upon the amount of the residue.

For neutral lipid analysis, the standard was Non-Polar Lipid Mixture-B (Materya Inc., Pleasant Gap, PA, USA), which contained 20.0% each of cholesterol (CH), oleic acid (OA), triolein (TO), methyl oleate (MO), and cholesteryl oleate (CO) with a total lipid concentration of 25.0 mg/mL. These compounds were used to represent free sterols, free fatty acids, triacylglycerols, methyl esters, and steryl esters, respectively. The standard was dissolved in a 25.0 mL volumetric flask in chloroform-methanol (2:1) to yield a final concentration of 0.200 µg/µL of each compound.

Analysis was performed on HPTLC silica gel 60 CF_{254} channeled silica gel plates (EMD Chemicals, Inc., Gibbstown, NJ, an affiliate of MerckKGaA, Darmstart, Germany), 10 × 20 cm, with a preadsorbent zone and 19 scored lanes. Plates were prewashed by development to the top with methanol and dried in a fume hood. Standards of neutral lipids were applied with a 10.0 µL Drummond (Broomall, PA, USA) digital microdispenser onto the preadsorbent of separate lanes in aliquots of 1.00, 2.00, 4.00, 8.00, and 16.0 µL, and reconstituted samples were applied in 2.00 µL–16.0 µL aliquots. Applied aliquots were allowed to dry for 30 s before development of the plate in a rectangular Camag (Wilmington, NC, USA) HPTLC twin-trough chamber that was lined with a saturation pad (Analtech, Newark, DE, USA) and allowed to equilibrate with the mobile phase for at least 15 min before inserting the plate. The mobile phase, petroleum ether-diethyl ether-glacial acetic acid (80:20:1), was allowed to reach a level 9 cm above the preadsorbent-silica gel interface, which required approximately 10 min. Developed plates were dried in a fume hood for 1 min using cool air from a hairdryer and sprayed with 5% ethanolic phosphomolybdic acid (PMA) solution. The plate was heated for at 115°C on a Camag plate heater until blue neutral lipid bands appeared on a yellow background.

The standard for polar lipid analysis, Polar Lipid Mix (Matreya, Inc., Pleasant Gap, PA, USA), contained 25.0% each of CH, phosphatidylethanolamine (PE), phosphatidylcholine (PC), and lysophosphatidylcholine (LPC) with a total of 25.0 mg of lipid. The standard was placed in a 25 mL volumetric flask and diluted with chloroform-methanol (2:1) to prepare a solution containing 0.0250 µg/µL of each lipid. HPTLC analysis of polar lipids was done on the same plates and with the same standard and sample volumes as described previously for the neutral lipids, but the mobile phase was chloroform-methanol-water (65:25:4) with a development time of approximately 20 min. The dried plates were sprayed with a 10% cupric sulfate solution and then heated at 140°C until brown bands on a white background were formed.

To quantify the amount of lipid in samples, a Camag TLC Scanner II was used with the following settings: tungsten light source at 610 nm for neutral lipids and deuterium light source at 370 nm for polar lipids, slit width 4, slit length 4, and scanning speed 4 mm/sec. The CATS-3 software was used to generate a linear regression calibration plot by relating the weights of the standard zones to their peak areas. From the calibration plot, the weights of the sample zones were automatically interpolated based on their measured peak areas. After the data was collected, if more than one aliquot of a single sample fell within the calibration curve, the weight corresponding to the sample area closest to the mean of the two middle standard areas was used for the calculation of lipid percent. The weight percentages of neutral and polar lipids in the whole snail DGGs were calculated using the following equation:

$$\% \text{ Lipid} = \frac{(w)(R)(F)(100)}{\mu g \text{ sample}}$$

Table 1

Weight percent (mean ± SE) of neutral and polar lipids in the DGGs of *B. glabrata* (Bg) *and H. trivolvis* (Ht) in 20% artificial ocean water (AOW) and deionized water (DI).

Snail	FS	TG	PC	PE
Ht in AOW	0.0766 ± 0.0243	0.239 ± 0.0407	0.6846 ± 0.135	0.201 ± 0.0343
Ht in DI	0.0742 ± 0.00504	0.153 ± 0.101	0.5973 ± 0.059	0.352 ± 0.099
Bg in AOW	0.0753 ± 0.0048	0.1697 ± 0.0621	0.8023 ± 0.311	0.4235 ± 0.0172
Bg in DI	0.0679 ± 0.0172	0.2214 ± 0.0776	1.288 ± 0.139	0.32234 ± 0.153

FS = Free sterols.
TG = Triacylglycerols.
PC = phosphatidylcholine.
PE = phosphatidylethanolamine.
n = 3 samples for each analysis.

where w = µg interpolated from the plot and R = [reconstituted volume (µL)]/[spotted volume (µL)]. For quantification of some samples, dilution or concentration was necessary in order to have scan area of at least one sample aliquot bracketed within the calibration plot. In these cases, a suitable factor (F) was included in the calculations.

RESULTS

Survival data was as follow: all snails (n = 4 for each species at each salinity) were dead at salinities of 100, 50, and 25% S within 90 min. A marked difference occurred at 20% salinity where 3 of 8 *B. glabrata* (37.5%) and 4 of 8 *H. trivolvis* (50%) survived the 2 week experimental period. Because of the relatively high survival in 20% salinity, it was this salt concentration that was selected for the lipid-HPTLC studies. Additional survival data showed that at 10% salinity, 6 of 8 (75%) *B. glabrata* and 7 of 8 (87.5%) *H.trivolvis* survived for 2 weeks. Controls in deionized water showed the following survival data at 2 weeks: 8 of 8 *B. glabrata* (100%) and 7 of 8 *H. trivolvis* (87.5%).

In the HPTLC studies, the major lipids quantified were free sterols (FSs), (TGs), PC and PE. The linear regression calibration plots relating the scan areas to weights of neutral lipid standard zones (0.400–3.20 µg) and polar lipids (0.500–4.00 µg) consistently gave linear regression correlation (r) values of 0.98 and 0.99, respectively.

Samples analyzed for neutral lipids showed zones that comigrated with standards with R_f values of 0.19 (CH) and 0.57 (TO) in every sample. Some samples had low amounts of free fatty acids that comigrated with the oleic acid standard at R_f 0.38. The faster moving neutral lipid sample zones, tentatively thought to be steryl esters and methyl esters, did not comigrate with CO and MO, respectively in the neutral lipid solvent system. These zones were relatively sparse in the snail samples, and since their identities were uncertain, they were not further characterized.

Samples analyzed for polar lipids showed zones with comparable migration to the standard with R_f values of 0.28 (PC) and 0.47 (PE). LPC was not able to be detected with the cupric sulfate reagent at the concentrations applied.

Table 1 shows the lipid percent ± standard error data of FS, TG, PC, and PE fractions of the DGGs of *H. trivolvis and B. glabrata* DGGs of snails maintained in 20% saline and DI water. The greatest concentrations in both species of snails were the phospholipids.

In spite of apparent differences in the means of certain values of some lipids in snails maintained in 20% saline versus DI water (for instance triacylglycerols from *H. trivolvis* snails , PC from *B. glabrata* snails and PE from *H. trivolvis* snails), Student's *t*-test ($P >$ 0.05) showed no intraspecific differences in the concentration of any lipids in snails maintained in the 20% saline solution versus those in DI water for the 2 week period.

DISCUSSION

Relatively few studies are available on the survival of fresh water pulmonates at various salinities. Of the pulmonate snails previously studied, *Lymnaea peregra* (Müller, 1774) was able to survive up to 10–11 parts per thousand (ppt) and *Physa fontinalis* (Linnaeus, 1758) survived up to 6 ppt (Hyman, 1967). Our study showed good survival of *H. trivolvis* and *B. glabrata* at 10 ppt and moderate survival of both species at 20 ppt. A marked reduction in survival was seen in both species at 25 ppt, compared to 20 ppt. We can offer no explanation at this time for such a dramatic difference in survival at 25 versus 20 ppt salinities.

The results of our HPTLC-lipid studies showed no significant changes in the concentrations of phospholipids and neutral lipid classes in the DGGs of *B. glabrata* and *H. trivolvis* maintained in DI versus 20%

saline. Our results on the effects of salinity on the lipid class composition in the DGGs of planorbids contrast noticeably to other studies, i.e., snail aging (Schneck et al., 2004), larval trematode parasitism (Bandstra et al., 2006b), and estivation and starvation (White et al., 2006), which showed significant increases or decreases in the concentration of one or more lipid classes in *B. glabrata* and *H trivolvis* snails as a function of the variable studied (see Bandstra et al., 2006a for a review). We conclude that adaptation of these snails to the different salinities used in our study did not interfere with the biochemistry of the lipid depot storage involving neutral lipids or the integrity of the structural lipids involving phospholipids.

Survival of *B. glabrata* in our study at 10 and even 20 ppt is of interest because de (da Silva et al., 2006) showed survival of this medically important snail from aquatic environments in coastal areas of Brazil at a salinity of 7.7 ppt. These authors were concerned that survival of *B. glabrata*, the vector of *Schistosoma masoni*, at such a relatively high salinity constitutes an increased risk for the spread of schistosomiasis in endemic waters where salinity may be elevated.

Although we did not monitor food intake or fecundity of our snails, we made incidental observations on feeding and egg laying under saline versus DI water conditions for both species of snails. Quantitatively, we found no differences in food intake or numbers of egg masses in our snail cultures maintained under saline versus DI conditions. Such findings indicate that these snails showed a reasonable tolerance to such relatively high salinities, at least under laboratory conditions. We were also surprised at how well both species of snails survived under experimental conditions for 2 weeks in DI. We have always used ASW as described by Ulmer (1970) to culture our planorbids (see Schneck & Fried, 2005 for a review). Perhaps for short term maintenance of *H. trivolvis* and *B glabrata*, the simpler medium of DI may be useful for snail maintenance as is the more complex ASW.

Acknowledgments. We would like to thank Dr. Charles Holliday for assistance in using the osmometer and Meredith White for assistance with the statistical analysis.

LITERATURE CITED

BANDSTRA, S. R., B. FRIED & J. SHERMA. 2006a. Effects of diet and larval trematode parasitism on lipids in snails as determined by thin-layer chromatography. Journal of Planar Chromatography—Modern TLC 19:180–186.

BANDSTRA, S. R., B. FRIED & J. SHERMA. 2006b. High-performance thin-layer chromatographic analysis of neutral lipids and phospholipids in *Biomphalaria glabrata* patently infected with *Echinostoma caproni*. Parasitology Research 99:414–418.

DA SILVA, P. B., C. S. BARBOSA, O. PIERI, A. TRAVASSOS & L. FLORENCIO. 2006. Physico-chemical and biological aspects related to the occurrence of *Biomphalaria glabrata* in foci of schistosomiasis in coastal areas of the state of Pernambuco, Brazil. Química Nova 29:901–906.

HYMAN, L. H. 1967. Pulmonata: physiology. P. 645 in The Invertebrates Volume VI Mollusca I. McGraw-Hill: New York, NY.

ONG, J. H. L., M. CHEJLAVA, B. FRIED & J. SHERMA. 2006. Effects of a hen's egg yolk diet on certain inorganic elements in the snail *Helisoma trivolvis* (Colorado strain). Veliger 48:1–7.

SCHNECK, J. L., B. FRIED & J. SHERMA. 2004. Effects of aging on the neutral and polar lipid composition of Biomphalaria glabrata under laboratory conditions. Veliger 47: 100–102.

SCHNECK, J. L. & B. FRIED. 2005. Growth of *Biomphalaria glabrata* (NMRI strain) and *Helisoma trivolvis* (Colorado strain) under laboratory conditions. American Malacological Bulletin 20:71–73.

ULMER, M. J. 1970. Notes on rearing snails in the laboratory. Pp. 143–144 in A. J. MacInnis & M. Voge (eds.), Experiments and Techniques in Parasitology. W.H. Freeman: San Francisco.

WHITE, M. M., B. FRIED & J. SHERMA. 2006. Determination of the effects of estivation and starvation on neutral lipids and phospholipids in *Biomphalaria glabrata* (NMRI strain) and *Helisoma trivolvis* (Colorado strain) snails by quantitative high performance thin layer chromatography-densitometry. Journal of Liquid Chromatography & Related Technologies 29:2167–2180.

WILLIAMS, W. D. 1987. Salinization of rivers and streams: an important environmental hazard. AMBIO 16:180–185.